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(54) **Stabilization of somatotropins and other proteins by modification of cysteine residues.**

(57) The present invention relates to physiologically-active derivatized natural and recombinant mammalian and human proteins and polypeptides. The invention provides chemical methods for derivatizing natural and recombinantly-derived proteins or polypeptides containing cysteine residues, either naturally or through site specific mutageneses. The pharmaceutical compositions containing said derivatized proteins and/or polypeptides are formulated to provide stable, long-acting compositions of such proteins and/or polypeptides, previously difficult to achieve.

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BACKGROUND OF THE INVENTION

The present invention relates to novel derivatized recombinant or natural proteins and/or polypeptides wherein if said protein and/or polypeptide contains one or more cysteine residue, said residue is derivatized with substituents as herein defined. Further, proteins not containing a cysteine in their natural forms can be altered, via site specific mutagenesis techniques, for instance, in order to introduce cysteines at advantageous sites and thereafter derivatize said cysteine(s).

Although molecular biological techniques have dramatically increased the availability of many proteins and/or polypeptides (hereinafter referred to as proteins), the therapeutic use of said proteins is often times hindered by other factors, such as aggregation, proteolytic degradation, poor bioavailability and physical properties which preclude efficient formulations. In the present invention the highly reactive sulfhydryl groups on cysteine residues within both recombinantly-derived and naturally-derived proteins are derivatized to modify the effective charge on the protein surface, and thereby the isoelectric point, or to link a surface-modifying polymer to the protein.

The advantage of modifying the charge isoelectric point is well known due to the minimization of protein aggregation if the pH of a protein formulation is significantly different than the isoelectric point. Manipulation of the isoelectric point, therefore, affords a technique to minimize aggregation.

Also, another mechanism for enhancing protein formulation is by conjugation of the protein with derivatizing compounds, which include, but are not limited to, polyethylene glycol and polypropylene glycol. Some of these benefits recognized include: lowered immunogenicity and antigenicity, increased duration of action, and altered pharmacokinetic properties. [F.M. Veronese, Chimicaoggi (1989) 53].

Heretofore, attachment of polymeric molecules to proteins was principally through lysine or other basic amino acids. This was accomplished with alkylating agents such as triazine derivatives, diazonium salts or active acylating agents such as succinic acid derivatives. These reactions were difficult to control and significant losses in biological activity occurred frequently due to reactions with essential amino acids in the proteins [see F. F. Davis et al Polymer (1979) 20 357].

In contrast, the present invention provides a selective method for modifying the less abundant cysteine residues either naturally occurring within a protein or incorporated within a protein by such methods as site specific mutogenesis.

It is an object of the present invention to provide compositions of derivatized proteins, which proteins include but are not limited to the (mammalian, avian and fish) somatotropins, mammalian IGF-1 and IGF-2, interleukins and interferons, prourokinase, tumor necrosis factor and antithrombin III which are biologically effective and yet stable to administer. Furthermore, as previously indicated, said proteins are not limited to those proteins which in their natural form contain cysteine residue(s) since site specific mutagenesis techniques are used to introduce cysteine at advantageous sites within said proteins. It is another object of the present invention to provide compositions of biologically active recombinant and/or natural proteins which are suitable for parenteral administration, said compositions comprising a biologically active amount of a derivatized somatotropin (animal or human form) or other protein or pharmaceutically and pharmacologically acceptable salt thereof in a pharmaceutically and pharmacologically acceptable solid or liquid carrier.

It is a further object of the present invention to provide novel compositions which will allow the modification of any cysteine-containing proteins. By judicious selection of the nature and number of ionizable-functional groups and/or the size and nature of the surface-modifying polymer a range of derivatized proteins with distinctly different physical properties are produced.

These and other objects of the invention will become more apparent by the more detailed description of the invention provided hereinbelow.

SUMMARY OF THE INVENTION

The present invention relates to novel derivatized recombinant or natural proteins which including, but not limited to somatotropins, IGF-1 and IGF-2, interleukins, interferons, prourokinase, tumor necrosis factor and antithrombin III, wherein at least one-naturally-occurring or incorporated cysteine residue within the protein is derivatized with substituents selected from ($CH_2CO_2R_1$), (CH_2CONHR_1), [$CH(COR_2)(CH_2)_x$-(COR_3)], [CH_2-$CONHCH(COR_2)(CH_2)_x(COR_3)$], or [$CH_2CONH(CH_2)_xCOR_4$]; wherein R_1 is CH_2CH_2-($OCH_2CH_2)_yOMe$; R_2 and R_3 are H or R_4; R_4 is $NHCH_2CH_2(OCH_2CH_2)_yOMe$ or $OCH_2CH_2(OCH_2CH_2)_yOMe$; X is an integer of from 1 to 3 and Y is an integer of from 10 to 300; with the proviso that R_2 and R_3 cannot simultaneously be H; provided that if the derivatized cysteine residue(s) of said protein formed a disulfide linkage prior to derivatization, both cysteines will bear the same sulfhydryl derivatives after the derivatization

procedure is complete. Furthermore, with some proteins containing more than one disulfide bond, it is possible to selectively cleave one or more of said disulfide bonds and those disulfide bonds which are selectively cleaved may be derivatized without affecting the remaining cysteine residues. When disulfides sequentially are cleaved it is possible to derivatize different cysteines with more than one composition. Furthermore, when said protein possesses an unpaired cysteine, that residues is derivatized without a reductive step.

The present invention also provides novel derivatizing compounds to said compounds comprising the following: $ZCH_2CO_2R_1$, $ZCH_2CONHCH(COR_2)[(CH_2)_x(COR_3)]$, $ZCH(COR_2)[(CH_2)_x(COR_3)]$ or $ZCH_2CONH(CH_2)_xCOR_2$ wherein R_1 is $CH_2CH_2(OCH_2CH_2)_yOMe$; R_2 and R_3 are H or R_4; R_4 is $NHCH_2CH_2(OCH_2CH_2)_yOMe$ or $OCH_2CH_2(OCH_2CH_2)_yOMe$; Z is Br or I; X is an integer of from 1 to 3 and Y is an integer of from 10 to 300; with the proviso that R_2 and R_3 can not simultaneously be H.

This invention further relates to a method for inhibiting aggregation of a recombinant animal somatotropin, by reducing at least one of the two disulfide bridges between the cysteine amino acid residues at the 55 and 166 positions or at the 183 and 191 positions of said somatotropins and thereafter derivatizing each of the cysteines of the reduced bridge or bridges at the 55 and 166 positions and/or at the 183 and 191 positions with the same derivative, provided that the derivatives on the cysteines at the 55 and 166 positions are the same or different substituents than the substituents on the cysteines at the 183 and 191 positions. The substituents employed in the preparation of the novel derivatized recombinant animal somatotropins of this invention include the following: $(CH_2CO_2R_1)$, $(CH_2CONHR_1)[CH(COR_2)(CH_2)_x(COR_3)]$, $[CH_2CONHCH(COR_2)(CH_2)_x(COR_3)]$, or $[CH_2CONH(CH_2)_xCOR_4]$; wherein R_1 is $CH_2CH_2(OCH_2CH_2)_yOMe$; R_2 and R_3 are H or R_4; R_4 is $NHCH_2CH_2(OCH_2CH_2)_yOMe$ or $OCH_2CH_2(OCH_2CH_2)_yOMe$; X is an integer of from 1 to 3 and Y is an integer of from 10 to 300; with the proviso that R_2 and R_3 can not simultaneously be H.

This invention further relates to a method to increase the half life of interleukin-2 by derivitizing the free cysteine at position 127 with the aforementioned derivatives.

In accordance with the present invention, the preferred novel animal somatotropins are recombinant porcine, bovine, ovine, human, avian and fish somatotropins wherein the disulfide bridge in the small loop of the somatotropin is reduced and the cysteines at the 183 and 191 positions derivatized with a substituent selected from (CH_2COR_1),
$[CH_2(CO_2R_2)(CH_2)_x(COR_3)]$,
$[CH_2CONHCH(COR_2)(CH_2)_x(COR_3)]$, or $[CH_2CONH(CH_2)_xCOR_4]$; wherein R_1, R_2, R_3, and X are as described.

Furthermore, another novel protein of the present invention is an animal somatotropin derivative in which cysteine residues are substituted for the Ser and Tyr residues at the 102 and 112 positions respectively and in which glutamic acid has been substituted for the cysteine residues which normally occur at positions 183 and 191. This protein is produced by site specific mutagenesis techniques which are well known to those skilled in the art. Furthermore, these techniques are utilized with other proteins to introduce a tethering site for polymers which does not interfere with or alter the ability of the hormone to interact with a binding site or to elicit a biological effect.

All of the plasmids, DNA sequences and microorganisms deposited in connection with the present patent application, except where specified to the contrary, are deposited in American Cyanamid Company culture collection maintained in Princeton, New Jersey and are deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland 20952, U.S.A.

DETAILED DESCRIPTION OF THE INVENTION

Preferred modified recombinant animal proteins of this invention include but not limited to animal and human somatotropins, interleukins and interferons, as illustrated hereinbelow. Recombinantly-derived somatotropins without the additional Asp-Gln additional substitutions or with other substitutions are prepared in accordance with the present invention, as well. Further animal proteins with deletions in amino acid chain length, additions to amino acid chain length, replacement of amino acids, (additional cysteines for derivatization), fragments with the active portion and the like are all within the scope of the present invention.

Recombinant Porcine Somatotropin

H-Met-Asp-Gln-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-
Leu-Phe-Ala-Asn-Ala-Val-Leu-Arg-Ala-Gln-His-Leu-
His-Gln-Leu-Ala-Ala-Asp-Thr-Tyr-Lys-Glu-Phe-Glu-
Arg-Ala-Tyr-Ile-Pro-Glu-Gly-Gln-Arg-Tyr-Ser-Ile-
55
Gln-Asn-Ala-Gln-Ala-Ala-Phe-Cys-Phe-Ser-Glu-Thr-
Ile-Pro-Ala-Pro-Thr-Gly-Lys-Asp-Glu-Ala-Gln-Gln-
Arg-Ser-Asp-Val-Glu-Leu-Leu-Arg-Phe-Ser-Leu-Leu-
Leu-Ile-Gln-Ser-Trp-Leu-Gly-Pro-Val-Gln-Phe-Leu-
Ser-Arg-Val-Phe-Thr-Asn-Ser-Leu-Val-Phe-Gly-Thr-
Ser-Asp-Arg-Val-Tyr-Glu-Lys-Leu-Lys-Asp-Leu-Glu-
Glu-Gly-Ile-Gln-Ala-Leu-Met-Arg-Glu-Leu-Glu-Asp-
Gly-Ser-Pro-Arg-Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-
Tyr-Asp-Lys-Phe-Asp-Thr-Asn-Leu-Arg-Ser-Asp-Asp-
166
Ala-Leu-Leu-Lys-Asn-Tyr-Gly-Leu-Leu-Ser-Cys-Phe-
Lys-Lys-Asp-Leu-His-Lys-Ala-Glu-Thr-Tyr-Leu-Arg-
183 191
Val-Met-Lys-Cys-Arg-Arg-Phe-Val-Glu-Ser-Ser-Cys-
Ala-Phe-OH.

Recombinant Bovine Somatotropin

H-Met-Asp-Gln-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-
Leu-Phe-Ala-Asn-Ala-Val-Leu-Arg-Ala-Gln-His-Leu-
His-Gln-Leu-Ala-Ala-Asp-Thr-Phe-Lys-Glu-Phe-Glu-
Arg-Thr-Tyr-Ile-Pro-Glu-Gly-Gln-Arg-Tyr-Ser-Ile-
55
Gln-Asn-Thr-Gln-Val-Ala-Phe-Cys-Phe-Ser-Glu-Thr-
Ile-Pro-Ala-Pro-Thr-Gly-Lys-Asn-Glu-Ala-Gln-Gln-
Lys-Ser-Asp-Leu-Glu-Leu-Leu-Arg-Ile-Ser-Leu-Leu-
Leu-Ile-Gln-Ser-Trp-Leu-Gly-Pro-Leu-Gln-Phe-Leu-
Ser-Arg-Val-Phe-Thr-Asn-Ser-Leu-Val-Phe-Gly-Thr-
Ser-Asp-Arg-Val-Tyr-Glu-Lys-Leu-Lys-Asp-Leu-Glu-
Glu-Gly-Ile-Leu-Ala-Leu-Met-Arg-Glu-Leu-Glu-Asp-

Gly-Thr-Pro-Arg-Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-
Tyr-Asp-Lys-Phe-Asp-Thr-Asn-Met-Arg-Ser-Asp-Asp-
166
Ala-Leu-Leu-Lys-Asn-Tyr-Gly-Leu-Leu-Ser-Cys-Phe-
Arg-Lys-Asp-Leu-His-Lys-Thr-Glu-Thr-Tyr-Leu-Arg-
183 191
Val-Met-Lys-Cys-Arg-Arg-Phe-Gly-Glu-Ala-Ser-Cys-
Ala-Phe-OH.

Recombinant Cys^{112,122}, Glu^{183,191} porcine somatotropin

H-Met-Asp-Gln-Phe-Pro-Ala-Met-Pro-Leu-Ser-Ser-
Leu-Phe-Ala-Asn-Ala-Val-Leu-Arg-Ala-Gln-His-Leu-
His-Gln-Leu-Ala-Ala-Asp-Thr-Tyr-Lys-Glu-Phe-Glu-
Arg-Thr-Tyr-Ile-Pro-Glu-Gly-Gln-Arg-Tyr-Ser-Ile-
Gln-Asn-Ala-Gln-Ala-Ala-Phe-Cys-Phe-Ser-Glu-Thr-
Ile-Pro-Ala-Pro-Thr-Gly-Lys-Asp-Glu-Ala-Gln-Gln-
Arg-Ser-Asp-Val-Glu-Leu-Leu-Arg-Phe-Ser-Leu-Leu-
Leu-Ile-Gln-Ser-Trp-Leu-Gly-Pro-Val-Gln-Phe-Leu-
112
Ser-Arg-Val-Phe-Thr-Asn-Cys-Leu-Val-Phe-Gly-Thr-
122
Ser-Asp-Arg-Val-Cys-Glu-Lys-Leu-Lys-Asp-Leu-Glu-
Glu-Gly-Ile-Gln-Ala-Leu-Met-Arg-Glu-Leu-Glu-Asp-
Gly-Ser-Pro-Arg-Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-
Tyr-Asp-Lys-Phe-Asp-Thr-Asn-Leu-Arg-Ser-Asp-Asp-
Ala-Leu-Leu-Lys-Asn-Tyr-Gly-Leu-Leu-Ser-Cys-Phe-
Arg-Lys-Asp-Leu-His-Lys-Thr-Glu-Thr-Tyr-Leu-Arg-
183 191
Val-Met-Lys-Glu-Arg-Arg-Phe-Val-Glu-Ser-Ser-Glu-
Ala-Phe-OH.

Recombinant Human Somatotropin

H-Met-Asp-Gln-Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-
Leu-Phe-Asp-Asn-Ala-Met-Leu-Arg-Ala-His-Arg-Leu-
His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Glu-

Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-
56
Leu-Gln-Asn-Pro-Gln-Thr-Ser-Leu-Cys-Phe-Ser-Glu-
Ser-Ile-Pro-Thr-Pro-Ser-Asn-Arg-Glu-Glu-Thr-Gln-
Gln-Lys-Ser-Asn-Leu-Glu-Leu-Leu-Arg-Ile-Ser-Leu-
Leu-Leu-Ile-Gln-Ser-Trp-Leu-Glu-Pro-Val-Gln-Phe-
Leu-Arg-Ser-Val-Phe-Ala-Asn-Ser-Leu-Val-Tyr-Gly-
Ala-Ser-Asp-Ser-Asn-Val-Tyr-Asp-Leu-Leu-Lys-Asp-
Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg-Leu-
Glu-Asp-Gly-Ser-Pro-Arg-Thr-Gly-Gln-Ile-Phe-Lys-
Gln-Thr-Tyr-Ser-Lys-Phe-Asp-Thr-Asn-Ser-His-Asn-
Asp-Asp-Ala-Leu-Leu-Lys-Asn-Tyr-Gly-Leu-Leu-Tyr-
167
Cys-Phe-Arg-Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-
184
Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-
191
Cys-Gly-Phe-OH.

Recombinant IL-2

H-Ala-Pro-Thr-Ser-Ser-Ser-Thr-Gly-Asn-Thr-Met-Lys-
Glu-Val-Lys-Ser-Leu-Leu-Leu-Asp-Leu-Gln-Leu-Leu-
Leu-Glu-Lys-Val-Lys-Asn-Pro-Glu-Asn-Leu-Lys-Leu-
Ser-Arg-Met-His-Thr-Phe-Asp-Phe-Tyr-Val-Pro-Lys-
Val-Asn-Ala-Thr-Glu-Leu-Lys-His-Leu-Lys-Cys-Leu-
Leu-Glu-Glu-Leu-Lys-Leu-Leu-Glu-Glu-Val-Leu-Asn-
Leu-Ala-Pro-Ser-Lys-Asn-Leu-Asn-Pro-Arg-Glu-Ile-
Lys-Asp-Ser-Met-Asp-Asn-Ile-Lys-Arg-Ile-Val-Leu-
Glu-Leu-Gln-Gly-Ser-Glu-Thr-Arg-Phe-Thr-Cys-Glu-
Tyr-Asp-Asp-Ala-Thr-Val-Asn-Ala-Val-Glu-Phe-Leu-
Asn-Lys-Trp-Ile-Thr-Phe-Cys-Gln-Ser-Ile-Tyr-Ser-
Thr-Met-Thr.

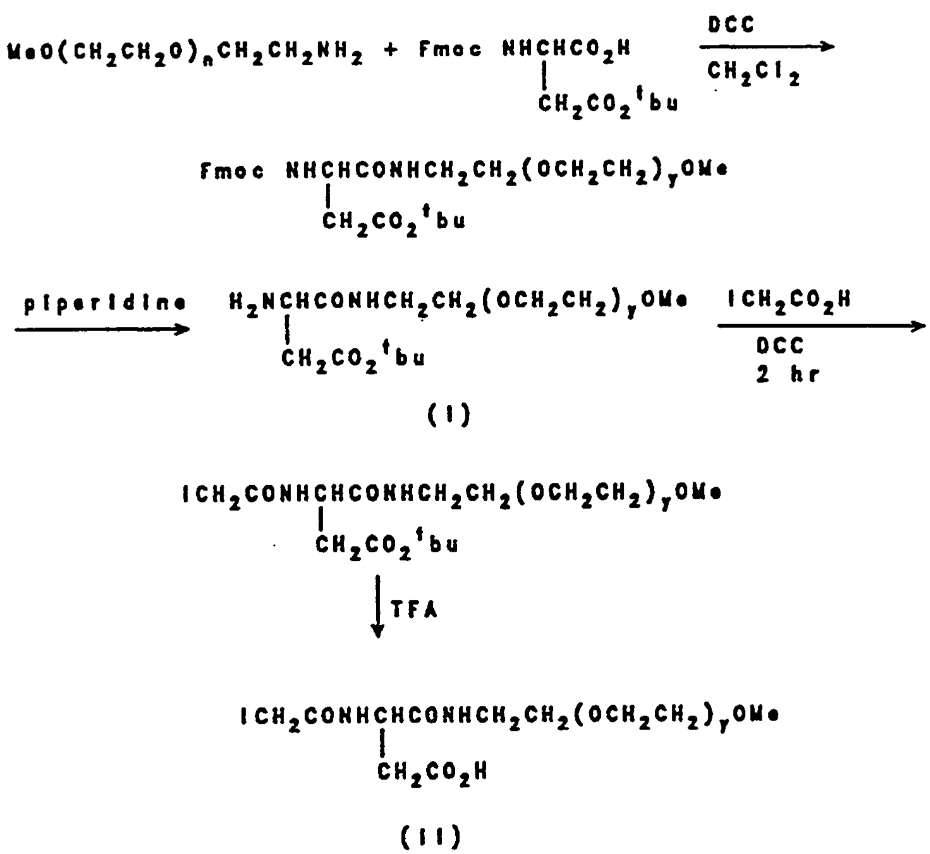
Derivatized Proteins

Derivatized proteins are prepared by dissolution or dispersion of protein in water or an aqueous solution of guanidine hydrochloride, sodium bicarbonate or the like, which is adjusted to pH 8.4 with an aqueous base, such as sodium or ammonium hydroxide. If reduction of a disulfide linkage is required, a dithiotheritol, i.e. (DL-threo-1, 4-dimer-capto-2,3-butanediol DTT) slowly is then added to this solution. The addition is

generally conducted under an atmosphere of nitrogen at room temperature. To the resulting solution is added the derivatizing agent. The mixture is stirred for about one to four hours. Progress of the reaction is monitored by Ellman titration of unmodified thiol groups or by gel electrophoresis. After the reaction is complete, it is desalted by ultrafiltration. The solution is concentrated and then subjected to several cycles of redilution with water, aqueous guanidine hydrochloride or the like, followed by ultrafiltration. The residue from the final filtration is then lyophilized to yield the derivatized protein.

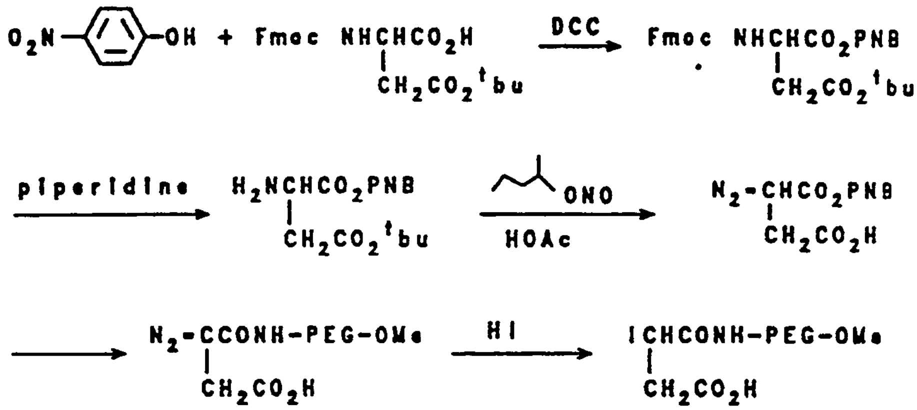
The preparation of two of the derivatizing agents proceeds from N-Fmoc-Asp(^{t}bu)OH and is depicted in Flow Chart 1 and Flow Chart 2. The amine derived from a polyethylene glycol monomethyl ether (PEG-OMe) is readily available using standard methodology. This amine is condensed with N-Fmoc-Asp(^{t}bu)OH using dicyclohexylcarbodiimide (DCC) or other related dehydrating agent. Removal of the Fmoc protecting group utilizing standard protocols affords (I). Condensation of (I) with iodoacetic acid is effected with DCC followed by removal of the tert-butyl ester affording the alkylating agent. It is readily apparent that similar derivatives in which the polymer is attached to only the b-carboxyl or both the a and b carboxyl groups are prepared depending upon the starting material used.

Flow Chart 1



The succinic acid derivative (Flow Chart II) also is prepared from N-Fmoc-Asp(^{t}bu)OH. The free acid is converted into the p-nitrobenzoate (PNB) ester with DCC and the amino group deprotected with piperidine.

Flow Chart 2



(III)

The amino group is converted to the diazonium salt (i-amyl nitrate/HOAc). Displacement of the active PNB ester with H_2N-PEG-OMe introduces the polymeric side chain. Treatment with HI results in simultaneous displacement of the diazo linkage with iodide and hydrolysis of the tert-butyl ester protecting group to afford the end result.

If N-protected 3-aminobutyric acid (Flow Chart 3) is utilized in place of Fmoc-N-Asp(^{t}bu)OA, the corresponding monofunctionalized iodoacetamide derivative is prepared. Likewise, PEG-iodoacetates are directly prepared by DCC-mediated coupling.

It should be apparent that the linkage of the polymer can be through either an amide or ester. These two functional groups have different susceptability to hydrolysis and the preferred mode of attachment will vary with the specific application. Furthermore, the PEG polymers are available in a variety of molecular weight ranges all of which are potentially useful. Also, the nature of the polymer need not be limited to polyethylene glycol or polypropylene glycol. Other potentially useful polymers include, but are not limited to, polyvinyl alcohol, dextrans and carbohydrates, polypyrrolidone.

Flow Chart 3

$$\text{Fmoc NH(CH}_2)_3\text{CO}_2\text{H+NH}_2\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_7\text{OMe} \xrightarrow[\text{CH}_2\text{Cl}_2 \text{ TFA}]{\text{DCC}}$$

$$\text{Fmoc NH(CH}_2)_3\text{CONHCH}_2\text{CH}_2(\text{CH}_2\text{CH}_2)_7\text{OMe}$$

$$\downarrow \text{piperidine}$$

$$\text{H}_2\text{N(CH}_2)_3\text{CONHCH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_7\text{OMe}$$

$$\xrightarrow[\text{DCC TFA}]{\text{ICH}_2\text{CO}_2\text{H}} \text{ICH}_2\text{CONH(CH}_2)_3\text{CONHCH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_7\text{OMe}$$

$$\text{MeO-(CH}_2\text{CH}_2\text{O)}_7\text{H+ICH}_2\text{CO}_2\text{H}$$

$$\text{DCC} \downarrow \text{CH}_2\text{Cl}_2 \text{ pyridine}$$

$$\text{ICH}_2\text{CO}_2\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_7\text{OMe}$$

Site Directed Mutagenesis

Preparation of a modified (substituted) recombinant animal (or human) somatotropins or other proteins of this invention can be achieved by site directed mutagenesis. Currently-utilized techniques for the alteration of the DNA sequence of a cloned segment of DNA at a specific defined site require the production of a single stranded form of that DNA. The single stranded DNA is annealed to a synthetic oligonucleotide which is complementary to a portion of it, except that the oligonucleotide contains within it a region of mismatch. The region of mismatch is usually located in the central portion of the oligonucleotide. The annealed mixture is then made double stranded and covalently closed by the addition of E. coli DNA polymerase I, large fragment and deoxynucleotide triphosphates in the presence of T4 DNA ligase and adenosine 5' triphosphate. The double stranded DNA is then transformed into an appropriate E. coli strain where the mismatched region of the DNA is repaired and replicated. Two opulations of clones are obtained. Dependant on which strand is chosen as the template for repair synthesis, a clone either contains the wild type or the altered (mutated) sequence. The clones which contain the mutated sequence, i.e. that which corresponds to the sequence of the oligonucleotide, are selected by hybridisation to the radioactively-labelled oligonucleotide. Due to the mismatch between the oligonucleotide and the wild type sequence, the radioactively-labelled oligonucleotide is more stably bound to the clones which contain the mutated sequence. Incubation at an appropriate temperature therefore differentiates between wild type and mutated clones. The alterations in the identified clones then are confirmed by DNA sequencing of the relevant regions.

One aim of mutagenesis is to generate a preferably-recombinantly-derived procein somatotropin (rpST) somatotropin molecule incapable of the formation of the small loop disulphide bond. The small loop disulphide is located between cysteines at 183 and 191. An additional aim is to incorporate two cysteine residues in the omega loop located at residues 102-112 which has been identified as a site of proteolytic cleavage. These newly incorporated cysteines are further modified using methods described herein. The numbering system is as described. The basic protocol used to obtain the required mutant is hereinafter described.

Substitution of the Ser102 and Tyr112 residues are achieved by using two oligonucleotides. The two oligonucleotides have the following sequence:

5' TTC ACC AAC TGT CTG GTG TTT 3' Ser102

5' GAC CGC GTC TGT GAG AAG CTG 3' Tyr112

The template single stranded DNA is pGEM3z(f+)pST-SX DNA. This single stranded DNA has already been modified to substitute two Glu residues for the cysteines normally found at positions 183 and 191 (This DNA is deposited in an expression vector PR0211, as indicated hereinbelow). 2000ng of single stranded PGEM3z(f+)pST-SX DNA is mixed with 50ng of AA1 oligonucleotide, which has previously been 5' phosphorylated with adenosine 5' triphosphate and polynucleotide kinase. The mixture is heated at 65°C for 7 minutes and then kept at room temperature for 10 minutes. This protocol anneals the oligonucleotide to the single stranded DNA. The annealed DNA is then converted to a double stranded covalently closed form by the addition of ATP, dNTP's (a mixture of the four deoxyribonucleotide 5' triphosphates), T4 DNA ligase and DNA polymerase I large fragment. The mixture is incubated for 1 hour at room temperature. The mixture is then transformed into HB101 competent cells by standard procedures. After overnight incubation at 37°C, colonies are transferred onto nitrocellulose filters and processed for hybridisation by standard protocols. Transformed colonies were identified using a radiolabelled oligonucleotide probe. Plasma DNA is prepared from these colonies, introduced into HB101 competent cell as described previously and re-screened with the radiolabelled oligonucleotide probe. Plasmid DNA is again prepared from colonies which hybridize with the probe. Plasmid DNA from one clone containing the S102C mutation, designated pGEM-3z(f+)pST-SXE-S102C is introduced into JM101 competent cells by transformation and single stranded DNA prepared from purified phage derived from a single transformant.

To mutate the tyrosine at position 112, a second oligonucleotide with following sequence is used:

5' GAC CGC GTC TGT GAG AAG CTG 3'

The process of mutagenesis is identical to that described above except that the template DNA is pGEM-3z(f+)pST-SXE-S102C (this is the clone isolated from the mutagenesis which has the serine at position 102 converted to cysteine) and glutamic acids substituted for cysteines at 183 and 191. The final wash temperature is 56°C. DNA sequencing reveals that the identified clones have the expected sequence. The mutated clone is designated pGEM-3z(f+)pST-SXE-S102C, Y112C.

The altered (mutant) clones are then reconstructed into the bacterial expression plasmid pR0211 (ATCC Accession Number 40483, deposited August 23, 1988) as described in Figure 5 of EPO publication 173,280. The pGEM clone is cut with EcoRI and HindIII and the pST gene fragment isolated. pRO211 is digested with the same enzymes treated with calf intestinal alkaline phosphatase and the large fragment isolated. The two pieces are ligated together with T4 DNA ligase and transformed into an appropriate bacterial strain, e.g. E. coli N99cI deposited.

In this strain a wild type g repressor is present. This prevents expression from the P_L promoter in pRO211. Once the appropriate construction is isolated, it is then transferred into bacterial strains which contain a temperature sensitive g repressor, e.g. (ATCC Accession Number 67766, deposted August 23, 1988) E. coli 4200. In these strains, the expression of pST is dependent on temperature. At 42°C, the repressor is inactive and expression occurs. At this stage, pST is prepared by procedures contained in EP 173,280 wherein expression occurs (incorporated herein by reference thereto). Expression of pST is not limited to these particular E. coli strains. Other strains with appropriate properties are substituted. The plasmid expression vectors are deposited with the ATCC. The bacterial strains are deposited separately.

Other amino acid substitutions can be made by the above procedures utilizing the appropriate codons and oligonucleotides. Similarly, these procedures are employed to modify a variety of animal or human somatotropins.

Method of Use

Advantageously, the novel animal proteins of the present invention are useful for alleviating disease states in animals; improving the growth rate of animals, especially meat producing animals; and increasing the efficiency of feed utilization thereby. Some of these compounds also are effective for enhancing the carcass quality of said animals, i.e. increasing the lean meat to fat ratio of said animals. Moreover, some of the compounds of the present invention are effective for increasing milk production in lactating animals and improving wool production in sheep and other animals raised for coats.

While the derivatized somatotropins of this invention are effective for treatment of animals to achieve the biological improvements described above, that the improved effectiveness and usefulness of the derivatized animal somatotropins of the invention attributed, in part, to the inhibition of aggregation of the somatotropin produced by the derivatization of said somatotropins. Such inhibition permits the preparation of markedly improved sustained release delivery systems which are not readily or effectively achieved with native somatotropins or recombinant somatotropins which are not derivatized as described by the present invention.

It is also found that the derivatized somatotropins of the present invention are highly stable and essentially free of aggregation due to dimer formation. Moreover, the derivatized somatotropins of the invention lend themselves to use in the preparation of significantly improved sustained release compositions.

Where native animal somatotropins have been found to aggregate in a single day, parenteral compositions containing the modified or derivatized somatotropins of this invention continue to release the modified or derivatized somatotropin for 10 days or more.

Compositions

In practice, the compositions of the present invention are generally administered to the animals by injection in the form of biologically active parenteral compositions. Among the parenteral compositions useful for administration of the recombinant animal proteins of this invention are gels, pastes, microspheres, microcapsules, implants and the like. As such, there is considerable interest in providing dosage forms of biologically active substances which release the substance in a controlled manner and thus, reduce the frequency of administration.

The development of sustained release compositions of biologically active macromolecules presents special problems due to their complex modes of action and intricate structures of the macromolecules. Thus, the development of effective sustained release compositions that contain the biologically active somatotropin is required.

The compositions useful for this type of administration are prepared by dissolving the modified or derivatized animal somatotropin in dilute ammonium hydroxide and then adding a solution of an alkali metal benzoate, laurate, carbonate or the like thereto. A nonionic surfactant is thereafter admixed with the solution and the resulting mixture spray dried. The thus formed solids are then admixed with molten fat or wax or a mixture thereof and the resulting molten mixture sprayed through an air/liquid spray nozzle equipped with a heated jacket to maintain the incoming air and the molten phase at a temperature above the melting point. The microspheres are formed as the molten droplets cool. These are collected on a series of sieves in the desired size range of about 45 to 180 microns and retained for use. Microspheres which are not of the desired size range are recycled. Alternatively, the homogeneous mixture is fed onto a centrifugal disc and the microspheres thus formed collected as above,. or the molten mixture are cooled and milled to the desired average particle size range.

The biologically active microspheres are then dispersed in a pharmaceutically and pharmacologically acceptable liquid vehicle for injection into the animal. The microsphere-liquid composition is generally administered by subcutaneous injection under the skin of the animal usually in the vicinity of the head, neck or ears.

The derivatized animal proteins of the present invention also are prepared as biocompatible implants which are injected under the skin of the animal using a conventional pellet implant gun. The compositions are prepared by admixing a powdered derivatized somatotropin with a wax such as castor wax or with a mixture of a copoly(glycolide/lactide), magnesium hydroxide, and a condensate of ethylene oxide prepared with a hydrophobic base formed by condensation of propylene oxide with propylene glycol. The thus formed compositions are then introduced into a pelleting press and formed into cylindrical pellets about 1/8 inch in diameter. The thus formed pellets are administered with a conventional pellet implant gun.

The present invention is further illustrated by the examples set forth hereinbelow.

EXAMPLE 1

Preparation of N-Fmoc-Asp(^{t}bu)NH-PEG-OMe

To solution of 1.24 g (3mM) of Fmoc Asp(^{t}bu)OH and 40 mL of CH_2Cl_2 is added 0.3 g (1.5 mM) of dicyclohexylcarbodiimide and the reaction mixture stirred at room temperature for one hour. The precipitated dicyclohexylurea is filtered off, and 3 g of polyethylene glycol (PEG) amine with an average molecular weight ($\overline{M}w$) of 2000 is added. The reaction is stirred overnight at room temperature. The methylene chloride is evaporated and the residue dissolved in 300 mL of H_2O. This solution was filtered, then ultrafiltered through a YM-2 Amicon membrane and lyophilized to afford 3 g of product.

EXAMPLE 2

Preparation of H_2N-Asp(^{t}bu)NH-PEG-OMe

3 g of Fmoc-NHAsp(ᵗbu)NH-PEG-OMe from Example 1 is added to 30 mL of piperidine-methylene chloride (1:1 v/v) at room temperature and stirred for 30 minutes. The methylene chloride is evaporated and the residue is dissolved in 200 mL of H_2O and ultrafiltered through an Amicon YM-2 membrane. The remaining aqueous solution is lyophilized to yield 2.5 g of product.

EXAMPLE 3

Preparation of $ICH_2CONHAsp(^tbu)NH$-PEG-OME

To a solution of 0.427 g of iodoacetic acid and 20 mL of methylene chloride is added 0.237 g of DCC and the reaction mixture is stirred at room temperature for one hour. The precipitated dicyclohexylurea is filtered and 2.5 g of H_2N-Asp(^{t}bu)NH-PEG-OMe is added and the mixture stirred for 3 hours at room temperature (until ninhydrin test is negative). The methylene chloride was evaporated and the residue dissolved in water and ultrafiltered through an Amicon YM-2 membrane. The solution is then lyophilized to yield 2.6 g of product.

EXAMPLE 4

Preparation of $ICH_2CONHAsp(OH)NH$-PEG-OMe

2.6 g of ICH_2CONH-Asp(^{t}bu)NH-PEG-OMe is dissolved in 40 mL of trifluoroacetic acid/methylene chloride (1:1 v/v) and the mixture is stirred at room temperature for one hour. The solvents are evaporated and the residue dissolved in about 200 mL of H_2O. The solution is ultrafiltered through an Amicon YM-2 membrane and neutralized at 0° with ammonium hydroxide. Lyophilization affords 2 g of $ICH_2CONHAsp$-(OH)NH-PEG-OMe.

EXAMPLE 5

Preparation of [(MeO-PEG-NHCOAsp(OH)NHCOCH$_2$)Cys ^{183,191}]-rpST

To a solution of 400 mg of r-pST in 200 mL of 0.5 M NH_4HCO_3 (pH=8.4) is added 28.0 mg of dithiothreitol and the reaction mixture stirred for 1 hour. To this reduced r-pST is added 1 g of MeO-PEG-NHCOAsp(OH)NHCOCH$_2$Iand the mixture allowed to stir for 3 hours. Ellman's test indicates free thiols remain, and so an additional one g of the iodoacetamide derivative was added and the reaction mixture stirred at room temperature for 18 hours. The reaction mixture is diluted, ultrafiltered through an Amicon YM-10 membrane and lyophilized to yield 400 mg of product.

EXAMPLE 6

Preparation of Fmoc NH Asp(^{t}bu)COPNB

To a solution of 8 g Fmoc-Asp(^{t}bu)OH and 250 mL of methylene chloride is added 2.01 gm of DCC and the resulting solution stirred at room temperature for 1.5 hours. The precipitated dicyclohexylurea was filtered and 1.35 g 4-nitrophenol is added and the reaction mixture stirred for 18 hours. The solution is cooled and washed with cold 5% Na_2CO_3 and brine. After drying, the solution is evaporated to yield 4.27 g of product.

EXAMPLE 7

Preparation of $H_2NCH(CO_2PNB)CH_2CO_2{}^tbu$

2 g of Fmoc $NHCH(CO_2PNB)CH_2CO_2{}^tbu$ was added to a 30 mL of an ice cold mixture of piperidine and methylene chloride (1:1 v/v) and stirred for 2 hours at 0°C. The soluents were evaporated and the residue redissolved in methylene chloride and washed with cold 0.1 N HCl, water and brine. The resulting solution was dried and evaporated to yield 0.83 g of product.

EXAMPLE 8

Preparation of $N_2CH(CONH\text{-}PEG\text{-}OMe)CH_2CO_2{}^tbu$

A solution of 2.0 g of $H_2NCH(CO_2PNB)CHCO_2$- ^{t}bu, 0.91 g i-amyl nitrite, 3 drops of HOAc and 100 mL of CH_2Cl were refluxed for 2 hours. The volatile solvents were evaporated and the residue redissolved in 50 mL of CH_2Cl_2 and 5 g of MeO-PEG-NH_2 were added. The mixture is stirred evernight at room temperature. The soluents were evaporated and the residue dissolved in H_2O and ultrafiltered through an Amicon YM-2 membrane. Lyophilization yielded 1.48 gm of product.

EXAMPLE 9

Preparation of $ICH(CONH\text{-}PEG\text{-}OMe)CH_2CO_2H)$

A solution of 0.5 g $N_2C(CONH\text{-}PEG\text{-}OMe)CH_2CO_2H$is dissolved in 50 mL of $CHCl_3$ and cooled to 0°C. 2 mL of HI is added carefully and the reaction stirred for 30 minutes at 0°C and then allowed to warm to 28°C for 2 hours. The solution is evaporated, dissolved in 30 mL H_2O and ultrafiltered through an Amicon YM-2 membrane. After lyophilization 0.3 g of product remains.

EXAMPLE 10

Preparation of $[(MeO\text{-}PEG\text{-}NHCOCH(CH_2CO_2H)Cys^{183,191})]$

To a solution of 500 mg of rpST in 250 mL of 0.5 N $NaHCO_3$ (pH = 8.4) is added 35 mg of dithiothreitol and the reaction mixture stirred for 1 hour. To the reduced rpST is added 2.0 g of MeO-PEG-NHCOCHI-(CH_2CO_2H) and the reaction mixture stirred overnight at room temperature. The reaction mixture is diluted with H_2O and ultrafiltered through an Amicon YM-10 membrane and lyophilized to yield 510 mg of product.

EXAMPLE 11

Preparation of MeO-PEG-$OCOCH_2I$

To a solution of 5 g of iodoacetic acid in 250 mL of methylene is added 2.76 g of DCC. The reaction mixture is stirred at ambient temperature for 2 hours and the precipitated dicyclohexylurea removed by filtration. To the remaining solution is added 20 g of MeO-PEG-OH and the reaction mixture stirred overnight at room temperature. The solution is then cooled and transferred to a separation funnel and extracted with 15 x 10 mL of cold saturated $NaHCO_3$. The remaining methylenechloride solution is evaporated and the residue dissolved in 30 mL of H_2O. This solution was ultrafiltered through an Amicon YM-2 membrane and finally lyophilized to yield 5.4 g of product.

EXAMPLE 12

Introduction of cysteines into the omega loop of porcine somatotropin using two synthetic oligonucleotides

A synthetic oligonucleotide designated S102C has the following sequence:
5' TTC ACC AAC TGT CTG GTG TTT 3'

This oligonucleotide differs from the analogous DNA sequence of the rpST gene in that the DNA encoding the serine codon at position 102 is replaced with DNA encoding cysteine. Therefore, the serine present at position 102 is replaced with cysteine.

Singe stranded pGEM-3z(f+)pST-SX DNA is the substrate for mutagenesis. This DNA is composed of a modified rpST gene contained in the commercially available phagemid, pGEM-3z(f+). The modifications of the rpST gene result in the alteration of the DNA sequence to allow for the introduction of a Sac I restriction endonuclease cleavage site at positions 225-230 of the coding region and for the introduction of an Xba I restriction endonuclease cleavage site at positions 285-290. These alterations in the DNA sequence do not change the amino acid sequence of the rpST protein. Two other modifications of the rpST gene result in the alteration of the DNA sequence to allow for the replacement of the cysteine residues at positions 183 and 191 each with glutamic acid. An EcoRI/HindIII fragment containing this modified rpST-SXE gene is cloned into pGEM-3z(f+) DNA cleaved with these restriction endonucleases using standard procedures, resulting in phagemid pGEM-3z(F+)pST-SX. The single stranded pGEM-3z(F+) DNA is

prepared from purified phage by standard protocols. 2000ng of this DNA is mixed with 100 ng of the C183del oligonucleotide, the latter of which has been phosphorylated at its 5' end previously with adenosine 5' triphosphate and polynucleotide kinase. The mixture is contained in a total volume of 10 ml in 1X annealing buffer (1X annealing buffer is 75 mM KCl and 5 mM Tris-Cl, pH 8.0). The mixture is heated at 65°C for 7 minutes followed by a 10 minute incubation at room temperature. This procedure permits the oligonucleotide to anneal to the single stranded substrate DNA. Annealed molecules are extended and converted to covalently closed, double stranded DNA by the addition of 22 ml H_2O, 1ml 20 mM ATP, 2 units each of T4 DNA ligase, and DNA polymerase I large fragment (for unit definition, see New England Biolabs catalogue, 1986), 2 ml 20 X dNTP's (a mixture of the four deoxyribonucleotide 5' triphosphates each at a concentration of 2 mM) and 4 ml 10X fill in buffer (1X fill in buffer is 27.5 mM Tris-Cl pH 7.5, 15 mM MgCl2, 2mM DTT). After a one hour incubation at room temperature, half of the reaction is introduced into HB101 competent cells by a standard transformation protocol. After overnight incubation at 37°C, colonies are transferred onto nitrocellulose filters and processed for hybridization by standard procedures. Oligonucleotide S102C is used for detection of the desired mutation by radiolabelling its 5' end with q-32P-ATP and polynucleotide kinase. After a three hour prehybridization at 37°C in 5X SSC, 1X Denhardt's and 150 mg/ml yeast tRNA, the radiolabelled oligonucleotide is added and allowed to hybridize with the DNA on the filters overnight at 37°C. The filters are washed for 30 minutes at 37°C in 5X SSC, followed by a 30 minute wash in TAC at 58.0°C. During this latter wash, only those colonies whose plasmid DNA contains the desired mutation will continue to hybridize with the radiolabelled oligonucleotide probe. These colonies are detected after exposure of the washed filters to X-ray film. Plasmid DNA is prepared from several of these colonies from the original petri plate, introduced into HB101 competent cells as described previously, and rescreened with the radiolabelled oligonucleotide probe as above. Plasmid DNA is prepared from colonies whose DNA hybridizes with the probe and analyzed for the desired DNA sequence. Plasmid DNA from one clone containing the S102C mutation, designated pGEM-3z(f+)pST-SXE-S102C, is introduced into JM101 competent cells by transformation and single stranded DNA prepared from purified phage derived from a single transformant.

To replace the tyrosine-encoding DNA at position 112 with cysteine-encoding DNA, the following oligonucleotide, designated Y112C, is synthesized:

5' GAC CGC GTC TGT GAG AAG CTG 3'

This oligonucleotide differs from the analogous DNA in the rpST gene in that the DNA encoding the tyrosine codon at position 112 is replaced by DNA encoding cysteine.

The template single stranded DNA from pGEN3z(f+)pST-SXE-S102C and Y112C oligonucleotide are annealed, extended and ligated as before. The mixture is introduced into HB101 competent cells and transformants are analyzed for the presence of the mutation exactly as described for S102C. The Y112C oligonucleotide is used as the radiolabelled detection probe. Hybridization and washing conditions are exactly as described for S102C. Several clones containing the Y112C mutation are identified from DNA sequence analysis. Plasmid DNA containing the S102C and Y112C mutations is designated pGEM-3z(f+)-pST-SXE-S102C, Y112C.

EXAMPLE 13

Reconstruction into appropriate bacterial expression plasmids

The altered (mutant) clones are reconstructed into the bacterial expression plasmid pRO211 (described in EP 173280), incorporated herein by reference thereto. The M13 clones are cut with ECoRI and HindIII and the pST gene fragment isolated. pRO211 is cloned with the same enzymes treated with calf intestinal alkaline phosphatase and the large fragment isolated. The two pieces are ligated together with T4 DNA ligase and transformed into an appropriate bacterial strain, e.g. N99cI*. In this strain a wild type g repressor is present. This prevents expression from the P_2 promoter in pRO211. Once the appropriate construction has been isolated, it is then transferred into bacterial strains which contain a temperature sensitive g repressor, e.g. 4200. In these strains, the expression of rpST is dependent on temperature. At 42°C, the repressor is inactive and expression occurs. At this stage, rpST can be prepared by procedures contained in EP 173280, incorporated herein by reference thereto. The E. coli strain which are used for expression of the pST gene product are not limited to E. coli 4200. Any appropriate E. coli strain can be utilized.

Following the procedures of Examples 1, 2 and 3 above, but substituting the appropriate codons for alanine, serine, glutamic acid, arginine, trypophan, or asparagine for cysteine at positions 183 and 191 converts TGT for cysteine to TCT, AGC or AGT for serine, GAG, or GAA for glutamic acid, CGN, AGA or AGG for arginine, AAT or AAC for asparagine, GCG for alanine, or TGG for tryptophan at said position.

EXAMPLE 14

Preparation of [(MeO-PEG-OCOCH$_2$)Cys^{102,112},Glu^{183,191}]rpST

To a solution of 200 mg of Cys^{102,112} Glu^{183,191} rpST in 150 mL of 0.5N NaH$_2$O$_3$ (pH=8.4) is added 28 mg of dithrothreital and the solution stirred for 1 hour. To the solution was added 500 mg of MeO-PEG-O$_2$CCH$_2$I($\overline{M}$w-550) and the reaction mixture stirred for 8 hours at room temperature. The reaction mixture is ultrafiltered through an Amicon YM-10 membrane to yield 135 mg of product.

EXAMPLE 15

Preparation of [(MeO-PEG-NHCOCH$_2$)Cys^{127}]Interleukin-2

To a solution of 25 mg of Interleukin-2 dissolved in 25 mL of 0.5N NaH$_2$O$_3$ (pH-8.4) is added 20 mg of MeO-PEG-NHCOCH$_2$I ($\overline{M}$w-2000) and the reaction mixture stirred for 48 hours until no free thiol is detected. The reaction mixture is ultrafiltered through an Amicon YM-10 membrance to yield 16 mg of product.

EXAMPLE 16

Preparation of [(MeO-PEG-OCOCH$_2$)Cys ^{183,191}]r-pST

To a solution of 400 mg of rpST in 250 mL of 0.5 N NaHCO$_3$ (pH=8.4) is added 28 mg of dithiothreitol and the solution stirred for 1 hour. To the solution is added 1.0 g of MeO-PEG-O$_2$CCH$_2$I ($\overline{M}$w=2000) and the reaction mixture stirred for 6 hours at room temperature. The reaction mixture is ultrafiltered through an Amicon YM-10 membrane and finally lyophilized to yield 420 mg of product.

EXAMPLE 17

Hypox rat test method for determining the growth enhancement of animals receiving derivatized recombinant animal somatotropin

The efficacy of various derivatized recombinant animal somatotropins of the present invention in altering the growth rate of animals is determined utilizing the standard hypophysectomized (hypox) rat assay. The hypophysectomized rat does not produce its own growth hormone and is sensitive to injected bovine growth hormone. The response measured is growth over a period of time, such as 10 days.

Each of the hypox albino rats (Taconic Farms, Sprague Dawley-derived) is injected with a sufficient quantity of the compositions prepared in accordance with the Xamples herein to provide a 10-day dose of 800 micrograms (80 micrograms/day) rPST in 0.2 ml of the formulation listed. Alternatively, each of the hypox albino rats is injected daily with 80 micrograms of rPST derivative.

Test Procedures

Prior to the test, the animals are weighed and the animals to be used for the test are selected based on body weight. Only those animals whose body weights are one standard deviation from the mean body weight of the group are selected. The resulting group is then randomly divided into treatment groups consisting of eight rats/group by a computer generated randomization procedure. The test animals are then transferred to a clean cage and housed four rats/cage. On the initial day of the study the test animals are weighed and any animals with excessive weight gain or loss (±5 grams) are replaced. The animals are then assigned to test groups and treated.

At the end of the ten-day test period, total weight gain for each animal is recorded and the average weight gain per rat for each treatment determined. The results of these experiments, which are summarized in Table I below, demonstrate the bioactivity of these derivatives.

TABLE I

Hypox Rat Test

Hypox Rat Data

derivative	PEG $\bar{M}W$	gms (growth) 0-2 days	2-4 days	4-7 days	7-10 days	total
[[(MeO-PEG-O_2CCH-($CH_2CO_2Na^+$)NHCO-CH_2]Cys183,191]-r-pST	2000	8.6	6.3	9.0	7.5	31.4
[(MeO-PEG-O_2C-CH_2)Cys183,191]-r-pST	5000	8.8	5.0	10.3	7.3	31.3
[(MeO-PEG-O_2C-CH_2)Cys183,191]-r-pST	2000	9.8	4.8	8.9	5.9	29.3
r-pST	-	6.9	5.3	6.8	9.0	28.0

From the above data it is seen that each of the derivatized recombinant porcine somatotropins evaluated is biologically active and provides excellent growth of animals.

EXAMPLE 18

Stability profiles of modified or derivatized recombinant animal somatotropins

A concentrated solution of the recombinant animal somatotropin derivative (up to 100 mg/ml) in phosphate buffered saline pH 7.4 (NaH_2PO_4 H_2O 3.45 gm, Na_2HPO_4 3.55 gm, NaCl 9.50 gm dissolved in distilled water to 1000 ml) is prepared. This is filtered through a millipore sterile Millex-0.22um filter unit and 0.1 ml aliquots placed into tubes. These are placed in a 45°C oven and removed at the required intervals. The contents are then diluted with phosphate buffered saline. The supernatent is assayed for monomer and dimer content by HPLC. A mass balance is done; any precipitated material is recorded. Results are compared with the initial concentrations, and a stability profile documented.

Alternately, a somatotropin derivative exhibiting poor solubility at pH 7.4 is dissolved at a less preferred pH (4-10) or is evaluated as a suspension (see Table II).

TABLE II

Sample		7 days Fraction Soln. (Dimer)	14 days Fraction Soln. (Dimer)	21 days Fraction Soln. (Dimer)
$[(MeO\text{-}PEG\text{-}OCO_2CH_2)Cys^{183,191}]$r-pST				
	$\bar{M}W$=2000	52	-	-
	$\bar{M}W$=5000	-	19	-
$[(MeO\text{-}PEG\text{-}NHCO)(CO_2^-Na^+)CHNHCOCH_2]Cys^{183,191}$ r-pST				
	$\bar{M}W$=200			83
r-pST technical		85.9(29.4)	65.4(45.5)	59.8(51.7)
		89.5(31.0)	64.0(44.9)	57.9(49.2)

EXAMPLE 19

Preparation of implants using $[(MeO\text{-}PEG\text{-}O_2CCH_2)Cys^{183,191})$ rpST and evaluation of said implants by in vitro dissolution

A set of implants are prepared from 96 mg of -270 mesh castorwax and 64 mg of powdered $[(MeO\text{-}PEG\text{-}O_2CCH_2)Cys^{183,191}]$rpST and rpST. The average molecular weight of the PEG is 2,000. The mixture is agitated, poured over a large surface petri dish and the CH_2Cl_2 allowed to evaporate at room temperature and then dried by vacuum drying. The dried residue is collected and formed in 1/8" diameter cylindrical implants using a Carver Press at about 1000 psig. The thus-formed implants weigh 60 to 70 mg each. The thus-prepared implants are then subjected to an in vitro dissolution evaluation. Each of the implants is placed in a separate plastic tube containing 10 ml of a phosphate buffer solution (ph 7.4 with 0.05% Na azide) and the tubes placed in a shaking water rack where the tubes are shaken while the temperature of the water in the unit is maintained at 39°C. The tubes are shaken for one day, then the solutions removed from each tube and analyzed for rpST by HPLC and the solution discarded. New phosphate buffer solution is added to each tube and the tubes shaken for three additional days thereafter. The solutions from each tube is again analyzed for rpST by HPLC and (the solution again is discarded). New phosphate buffer are again added to each tube and the tube again shaken for three days then analyzed again for rpST.

The phosphate buffer solution is ($NaH_2\text{-}PO_4$ H_2O 3.45 g, Na_2HPO_4 3.55 g, Nacl 9.5 g dissolved in distilled water to 1000 ml (see Table III).

TABLE III

Implant Release
Implants pressed from a mixture of 60% Castorwax and 40% r-pST and coated with silicone tubing

Compound	PEG Mw	% Released Day 1	Day 4	day 7
r-pST		8.1	11.3	12.9
[(MeO-PEG-OCCH$_2$)-Cys^{183,191}]pST	2000	12.4	17.1	17.6

Claims

1. A derivatized protein or polypeptide wherein at least one cysteine residue of said protein or polypeptide is derivatized with substituents, said substituents comprising: ($CH_2CO_2R_1$), (CH_2CONHR_1), [$CH(COR_2)$-$(CH_2)_x$ (COR_3)], [$CH_2CONHCH(COR_2)$-$(CH_2)_x(COR_3)$] or [CH_2CONH-$(CH_2)_xCOR_4$]; wherein $R_1 = CH_2$-$(OCH_2(CH_2CH_2)_yOMe$; R_2 and R_3 are H or R_4; $R_4 = NHCH_2CH_2(OCH_2CH_2)_yOMe$ or $OCH_2CH_2(OCH_2$-$CH_2)_yOMe$; wherein X is an integer of from 1 to 3 and Y is an integer of from 10 to 300; with the proviso that R_2 and R_3 cannot simultaneously be H; provided that when the derivatized cysteine residue(s) of said protein or polypeptide form, a disulfide linkage prior to derivatization, both cysteines will bear the same sulhydryl derivative; and when the protein is somatotropin, said substituted on the cysteine is not CH_2CONHR_1.

2. A protein or polypeptide according to Claim 1, wherein said protein or polypeptide contains at least one cysteine residue in its natural form.

3. A protein or polypeptide according to Claim 1, wherein said protein or polypeptide has had a cysteine residue added or substituted in the protein or polypeptide natural form.

4. A protein or polypeptide according to Claim 2, wherein said protein or polypeptide comprises: somatotropins, interleukins, interferons, prourokinases IGF-Is, IGF-2s, growth factors, such as fibroblast growth factor, and antithrombin III.

5. A protein or polypeptide according to Claim 3, wherein said protein or polypeptide comprises somatotropins, interleukins, interferons, prourokinases IGF-Is, IGF-2s, growth factors and antitrobin III.

6. A protein according to Claim 4, wherein said protein is somatotropin.

7. A protein according to Claim 5, wherein said protein is somatotropin.

8. A protein according to Claim 6, wherein said protein is Cys^{102,112} Glu^{183,191},pST or Glu^{183,191}pST.

9. A protein according to Claim 4, wherein said protein is interleukin-2.

10. A protein according to Claim 5, where said protein is interleukin-2.

11. The compounds comprising: $ZCH_2CO_2R_1$, $ZCH_2CONHCH$ (COR_2)-[$(CH_2)_x(COR_3)$], [$(_cH_2)_x(COR_3)$] or ZCH CONH-($CH_{2x}COR_2$ hwerein $R_1 = CH_2ch_2(OCH_2CH_2)_yOMe$;$R_2$ and R_3 are H or R_4;

R_4 = $NHCH_2CH_2(OCH_2CH_2)_yOMe$ or $OCH_2CH_2(OCH_2$-$CH_2)HyHOMe$; Z is Br or I; X is an integer of from 1 to 3 that R_2 and R_3 can not simultaneously be H.

12. A compound $KH_2CO_2R_1$ according to Claim 11, wherein Y is about 10 to 100.

13. A compound $KHCOR_2$ according to Claim 11, wherein R_2 Y is an integer from 10 to 100.

14. A compound $KH_2OONH(COR_2)[CCH_2)_xCOR_3)]$ according to Claim 11, wherein Y is an integer of about 1 to 2 and Y is about 10 to 100.

15. A compound $ICH_2CONH(CH_2)_xCOR_2$ according to Claim 11, wherein X is is an integer about 1 to 3 and Y is an integer about 10 to 100.

16. A pharmaceutical composition comprising: a pharmacologically effective amount of a modified or derivatized protein or polypeptide or a pharmaceutically acceptable salts thereof; and a pharmaceutically acceptable solid or liquid carrier therefore, wherein said composition is effective in increading the growth rate of an animal over an extended period of time.

17. A composition according to Claim 16, wherein said composition is parenterally administered to said animal.

18. A protein according to Claim 4, wherein said protein is fibroblast growth factor.

19. A protein according to Claim 5, wherein said protein is fibroblast growth factor.